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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/883,343	06/19/2001	Michael E. Stiles	C60007.1US	1463

7590 05/09/2006

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EXAMINER	
MOSHER, MARY	

ART UNIT	PAPER NUMBER
1648	

DATE MAILED: 05/09/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/883,343	STILES ET AL.	
	Examiner	Art Unit	
	Mary E. Mosher, Ph.D.	1648	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *03 September 2003*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *18,20 and 37-49* is/are pending in the application.

4a) Of the above claim(s) *18,20 and 41-49* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *37-40* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *19 June 2001* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Priority

It is noted that this application appears to claim subject matter disclosed in prior Application No. 08/924629, filed 9/5/1997. A reference to the prior application must be inserted as the first sentence(s) of the specification of this application or in an application data sheet (37 CFR 1.76), if applicant intends to rely on the filing date of the prior application under 35 U.S.C. 119(e), 120, 121, or 365(c). See 37 CFR 1.78(a). For benefit claims under 35 U.S.C. 120, 121, or 365(c), the reference must include the relationship (i.e., continuation, divisional, or continuation-in-part) of all nonprovisional applications. Since the reference to the prior application was previously submitted within the time period set forth in 37 CFR 1.78(a), but not in the first sentence(s) of the specification or an application data sheet (ADS) as required by 37 CFR 1.78(a), and the information concerning the benefit claim was recognized by the Office as shown by its inclusion on the first filing receipt, a petition under 37 CFR 1.78(a) and the surcharge under 37 CFR 1.17(t) are not required. Applicant is still required to submit the reference in compliance with 37 CFR 1.78(a) by filing an amendment to the first sentence(s) of the specification or an ADS. See MPEP § 201.11.

Please note, this is also required to establish co-pendency with provisional application 60/026257.

In the interest of compact prosecution, this application has been treated as if it properly claimed benefit of priority to the above two applications. However, this

treatment does not relieve applicant of the burden of amending the specification or filing an ADS with the required information.

Election/Restrictions

Applicant's election of group I, species bacteriocin with divergicin A processing polypeptide, in the reply filed on 9/3/2003 is acknowledged. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Claims 18, 20, 41-47 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected species, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on 9/23/2003.

Claim Rejections - 35 USC § 112

Claims 38-40 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claim 39 is confusing because it lacks antecedent for "said heterologous bacteriocin". Claim 38 is indefinite because of the recitation "an operable mutein thereof". The specification does not indicate what characteristics distinguish an operable mutein of the divergicin A processing peptide from any operable signal peptide or leader peptide. Therefore the metes and bounds of the claimed subject matter are unclear, and the claim is indefinite.

Claims 38-40 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to

one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a "written description" rejection, directed at the genus of "operable muteins" recited in the claims. The specification fully describes the structure of the signal peptide of divergicin A. However, the specification does not teach the structure of any "operable muteins thereof", nor teach what parts of the structure are important for operability. Considering the unpredictable effects of any sequence change upon the function of this polypeptide, and the lack of any art-recognized correlation between the amino acid sequence and the functional activity of this polypeptide, it is concluded that the specification does not reasonably convey that applicants possessed the "operable muteins" recited in the claims.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 37-39 are rejected under 35 U.S.C. 102(a) as being anticipated by McCormick et al (Applied and Environmental Microbiology 62:4095-4099, 11/96). The reference teaches inhibiting the growth of susceptible bacteria in an environment, using cells transformed with a n expression vector encoding divergicin A signal peptide fused to the bacteriocin carnobacteriocin B2, and the immunity gene for the bacteriocin, see for example page 4097, first paragraph, Figure 2, and Table 2. The reference therefore meets each and every limitation of the claims.

Claims 37-39 are rejected under 35 U.S.C. 102(b) as being anticipated by Worobo et al (Journal of Bacteriology 177:3143-3149, 6/95). The reference teaches inhibiting the growth of susceptible bacteria in an environment, using cells transformed with an expression vector encoding divergicin A signal peptide, divergicin A bacteriocin, and divergicin immunity, see for example pRW5.6 transformants in Figure 3. The reference therefore meets each and every limitation of the claims.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claim 40 is rejected under 35 U.S.C. 103(a) as being unpatentable over either McCormick et al (Applied and Environmental Microbiology 62:4095-4099, 11/96) or Worobo et al (Journal of Bacteriology 177:3143-3149, 6/95). On page 4099, McCormick

et al suggests production of lactic acid bacteria producing bacteriocins, combinations of bacteriocins, and use of the divergicin A signal peptide for expressing various bacteriocins from a single organism. Although the reference does not explicitly suggest placing the genes for two bacteriocins in the same vector, it would have been readily apparent to one of ordinary skill that it would be advantageous to place genes encoding a combination of different bacteriocins in a single vector. On page 3143 of Worobo et al, the reference discusses production of two or more bacteriocins within gene cassettes, and on page 3148 suggests fusions of the divergicin signal peptide with other bacteriocins for secretion without specific secretion and maturation proteins. It would have been within the ordinary skill of the art to carry out the suggestions made in the reference, to fuse the divergicin signal peptide to the structural gene of another bacteriocin in a gene cassette, and to combine two gene cassettes to produce two pr more bacteriocins from a single vector. The invention as a whole is therefore prima facie obvious, absent unexpected results.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 37-40 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-3 of U.S. Patent No. 6403082. Although the conflicting claims are not identical, they are not patentably distinct from each other because the application claims (1) encompass the subject matter of the patented claims and/or (2) are drawn to embodiments of the patented subject matter which are obvious embodiments when the patent claims are viewed in light of the supporting disclosure.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Mary E. Mosher, Ph.D. whose telephone number is 571-272-0906. The examiner can normally be reached on Monday-Thursday and alternate Fridays.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Housel can be reached on 571-272-0902. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

5/5/06

MARY E. MOSHER, PH.D.
PRIMARY EXAMINER

Notice of References Cited	Application/Control No. 09/883,343	Applicant(s)/Patent Under Reexamination STILES ET AL.	
	Examiner Mary E. Mosher, Ph.D.	Art Unit 1648	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,403,082	06-2002	Stiles et al.	424/93.2
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	McCormick et al (Applied and Environmental Microbiology 62:4095-4099, 11/96)
	V	Worobo et al (Journal of Bacteriology 177:3143-3149, 6/95).
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1996, p. 4095–4099 Vol. 62, No. 11
0099-2240/96/$04.00+0

Expression of the Antimicrobial Peptide Carnobacteriocin B2 by a Signal Peptide-Dependent General Secretory Pathway

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Received 30 August 1996/Accepted 11 September 1996

Carnobacteriocin B2 is a well-characterized class II bacteriocin produced by a 61-kb plasmid from *Carnobacterium piscicola* LV17. Export of this bacteriocin is dependent on specific ABC (ATP-binding cassette) secretion proteins. Divergicin A is a strongly hydrophobic narrow-spectrum bacteriocin produced by a 3.4-kb plasmid from *Carnobacterium divergens* LV13. Predivergicin A contains a signal peptide and utilizes the general secretary pathway for export (R. W. Worobo, M. J. van Belkum, M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles, J. Bacteriol. 177:3143–3149, 1995). Fusion of the carnobacteriocin B2 structural gene (devoid of its natural leader peptide) behind the signal peptide of divergicin A in the expression vector pMG36e permitted production and export of active carnobacteriocin B2 in the absence of the specific secretion genes. N-terminal sequencing of purified carnobacteriocin B2 established that correct processing of the prebacteriocin occurred beyond the Ala-Ser-Ala cleavage site of the signal peptide. Carnobacteriocin B2 was produced by the wild-type strain of *C. divergens*, LV13, and in *C. piscicola* LV17C, the nonbacteriocinogenic, plasmidless variant of the original carnobacteriocin B2 producer strain. The corresponding immunity gene was included immediately downstream of the structural gene. Both of the host strains are sensitive to the bacteriocin, and both acquired immunity when they were transformed with the construct. *C. divergens* LV13 containing the divergicin A signal peptide-carnobacteriocin B2 fusion construct produces both divergicin A and carnobacteriocin B2 and demonstrates the first example of multiple-bacteriocin expression via the general secretory pathway. The small amount of genetic material required for independent bacteriocin expression has implications for the development of a food-grade multiple-bacteriocin expression vector for use in lactic acid bacteria.

Bacteriocins of lactic acid bacteria (LAB) are antimicrobial, proteinaceous compounds which typically kill or inhibit growth of closely related bacteria. They were classified into four categories by Klaenhammer (18). Class I and II bacteriocins are the best characterized, and they are distinguished as small, heat-stable peptides. Class I bacteriocins, also termed lantibiotics, undergo extensive posttranslational modification which results in the formation of lanthionine and β-methyllanthionine thioether ring structures from the condensation of cysteine with the unusual amino acids dehydroalanine and dehydrobutyrine, respectively. Class II bacteriocins do not undergo posttranslational modification except for cleavage of the leader peptide and, in some cases, formation of disulfide bridges. Class III and IV bacteriocins are the least well characterized and they are distinguished as large, heat-labile proteins and protein complexes containing other chemical moieties, respectively.

Well-characterized class II bacteriocins possess an N-terminal extension of typically 18 to 24 amino acids containing a conserved Gly^{-2}-Gly^{-1}-Xaa^{+1} processing site (15, 18). There is a growing family of class II bacteriocins, including carnobacteriocins A (36), B2, BM1 (26, 27), and leucocin A (13, 31). Colicin V, produced by many strains of *Escherichia coli*, is a class II bacteriocin (11, 15). For some class II bacteriocins (3, 14, 23, 25, 31) and colicin V (10, 11), it has been shown that export of the bacteriocin from the cell is contingent upon the presence of an ATP-dependent membrane translocator protein, also termed an ATP-binding cassette (ABC) exporter, and an accessory protein whose function in gram-positive-bacterium systems is currently unknown (8). The genes encoding these proteins are generally located in close proximity to the structural and immunity genes of the bacteriocins. The ABC transporter has also been shown to function as the maturation protease responsible for cleavage of the N-terminal leader peptide at the conserved Gly^{-2}-Gly^{-1}-Xaa^{+1} processing site for lactococcin G (14) and pediocin PA1.0 (33).

In contrast to bacteriocins and other members of the ABC transporter family, most exported prokaryotic proteins contain an N-terminal signal peptide which is necessary for their export via a common general secretory pathway (the SEC pathway) (for reviews see references 24 and 30). Worobo et al. (37) characterized divergicin A, a strongly hydrophobic, narrow-spectrum bacteriocin from *Carnobacterium divergens* LV13 that is novel in that export of the bacteriocin is not dependent on dedicated secretion machinery. Unprocessed divergicin A contains a leader peptide that does not contain the typical Gly-Gly processing site and does not have homology with the leader sequences of class II bacteriocins; however, it does display characteristics of a prokaryotic signal peptide (37). Fusion of the signal peptide of divergicin A to alkaline phosphatase resulted in secretion of the enzyme into the periplasmic space in *E. coli* (37). Leer et al. (21) characterized acidocin B, a bacteriocin produced by *Lactobacillus acidophilus*, in which bacteriocin expression also appears to be SEC dependent. Furthermore, export of colicin V into the periplasm of *E. coli* has been accomplished by fusion of the OmpA signal peptide in front of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
C. divergens LV13	*dvn*$^+$ *dvi*$^+$ (containing pCD3.4), CbnB2^s	NCFB[b,c]
C. piscicola LV17C	Dvn^s CbnB2^s, plasmidless	1
E. coli		
DH5α	F *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ *recA1 gyrA96 relA1* Δ(*argF*− *lacZYA*) UI69φ80*dlacZ*ΔM15	BRL Life Technologies Inc.
MH1	MC1061 derivative; *araD139 lacX74 galU galK hsr hsm*$^+$ *strA*	6
Plasmids		
pUC118	*lacZ'* Amp^r 3.2 kb	34
pMG36e	Expression vector, Em^r, 3.6 kb	32
pLQ24	pCaT containing 16-kb insert from pCP40, *cbnB2*$^+$ *cbiB2*$^+$ Cm^r, 24.5 kb	27
pCD3.4	*dvn*$^+$ *dvi*$^+$ (divergicin A producer), 3.4 kb	37
pRW19e	pMG36e containing 514-bp *Eco*RV-*Acc*I fragment; *dvn*$^+$ *dvi*$^+$ Em^r	This study
pJKM05	528-bp *Hin*dIII-*Xba*I Δ*cbnB2*$^+$ *cbiB2*$^+$ PCR product in pUC118, Amp^r	This study
pJKM14	pRW19e containing 528-bp *Hin*dIII-*Xba*I fragment from pJKM05, *dvn-cbnB2+ cbiB2*$^+$ Em^r	This study
pJKM16	335-bp *Sac*I-*Eco*RI fragment from pJKM14 cloned in pUC118, Amp^r	This study

[a] *dvn*$^+$, divergicin A structural gene; *dvi*$^+$, divergicin A immunity gene; *cbnB2*$^+$, carnobacteriocin B2 structural gene; *cbiB2*$^+$, carnobacteriocin B2 immunity gene; Δ*cbnB2*$^+$, carnobacteriocin B2 structural gene with sequence encoding leader peptide truncated; *dvn-cbnB2*$^+$, sequence encoding divergicin A signal peptide fused to carnobacteriocin B2 structural gene; Dvn^s, divergicin A susceptible; CbnB2^s, carnobacteriocin B2 susceptible; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant.

[b] NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

[c] Supplied by B. G. Shaw, Institute for Food Research, Langford, Bristol, United Kingdom.

the mature structural gene for colicin V (38). Export of colicin V across the outer membrane did not occur.

This study reports the fusion of the signal peptide from divergicin A in place of the leader peptide of carnobacteriocin B2 to allow export of active bacteriocin in the absence of its dedicated secretion machinery. The carnobacteriocin B2 immunity gene was included immediately downstream of the structural gene. Carnobacteriocin B2 was produced in the non-bacteriocinogenic plasmidless variant of the original carnobacteriocin B2 producer strain *Carnobacterium piscicola* LV17C and by the wild-type strain *C. divergens* LV13. N-terminal amino acid sequencing of purified carnobacteriocin B2 established that proper processing of the prebacteriocin occurred. Carnobacteriocin B2 produced in *C. divergens* LV13 demonstrates the first example of multiple-bacteriocin expression via the general secretary pathway.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. Carnobacteria were grown in APT broth (Difco Laboratories, Detroit, Mich.) at 25°C without agitation. *E. coli* was grown in Luria-Bertani medium at 37°C on a rotary shaker (29). Agar plates were made by addition of 1.5% (wt/vol) agar to broth media. Antibiotics were added as selective agents when appropriate: erythromycin (200 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) for *E. coli* and erythromycin (5 μg ml^{-1}) for carnobacteria. Stock cultures of the bacterial strains were stored at −70°C in the appropriate broth containing 20% (vol/vol) glycerol.

Oligonucleotide primer synthesis and amplification reactions. In the 3' region of the nucleotide sequence encoding the signal peptide of divergicin A there is a *Hin*dIII restriction site located 10 nucleotides upstream of the sequence encoding mature divergicin A (37). A 35-mer oligonucleotide designed to facilitate an in-frame fusion between the signal peptide of divergicin A and the structural gene of carnobacteriocin B2 was synthesized on a DNA synthesizer (Applied Biosystems 391 PCR Mate) for use as a PCR primer (JMc7: 5'-CCCAAGCTT CTGCTGTAAATTATGGTAATGGTGTT-3'). The first 9 nucleotides of JMc7 regenerate the *Hin*dIII restriction endonuclease cleavage site followed by nucleotides encoding the carboxy terminus of the divergicin A signal peptide. The last 21 nucleotides of the primer are complementary to the 5' sequence corresponding to the N-terminal sequence of the carnobacteriocin B2 structural gene (*cbnB2*) immediately after the Gly-Gly cleavage site of the leader peptide. The reverse primer for the PCR amplification (ImmR) was based on the 3' nucleotide sequence of the immunity gene for carnobacteriocin B2 (*cbiB2*) and contains an overhang of 9 nucleotides to accommodate an *Xba*I restriction endonuclease site (27). DNA was amplified in a 100-μl reaction mixture by using a temperature cycler (OmniGene; InterSciences Inc., Markham, Ontario, Canada). PCR mixtures contained 1.0 μM each primer, 200 μM deoxynucleoside triphosphates, 5 mM $MgCl_2$, 2.5 U of *Tli* DNA polymerase (Promega), and 1× reaction buffer (Promega). pLQ24 (27) was used as template DNA for the carnobacteriocin B2 reaction. DNA was amplified with 36 cycles (denaturation, 93°C, 1 min; annealing, 48°C, 1 min; extension, 75°C, 2 min) followed by a final extension step at 75°C for 5 min.

DNA isolation and manipulation and sequence determination. Isolation of plasmid DNA from *E. coli* and carnobacteria was done as previously described (29, 36). Standard methods were used for restriction enzyme digestion, ligations, gel electrophoresis, and *E. coli* transformation (29). Transformation of carnobacteria was done as described by Worobo et al. (37). DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing (Applied Biosystems model 373A). Sequences were determined bidirectionally in pUC118 by using universal primers.

Production of and immunity to divergicin A and carnobacteriocin B2. Carnobacteria transformed with either pMG36e, pRW19e or pJKM14 were tested for bacteriocin production by using the deferred-antagonism assay (1). Strains containing pMG36e were used as negative controls. The amount of carnobacteriocin B2 produced from strains containing pJKM14 was determined by the spot-on-lawn test (1) at 3-h intervals from a 0.1% inoculum of an overnight culture incubated at 25°C. *C. divergens* LV13 containing pMG36e was used as the indicator organism. Immunity was determined with the transformants as indicators in deferred-inhibition assays. To confirm that the zones of inhibition were caused by a proteinaceous compound, they were inactivated by spotting Pronase E (1 mg ml^{-1}; Sigma) prior to overlayering with the sensitive indicator strain.

Purification and N-terminal sequencing of carnobacteriocin B2. Partial purification of carnobacteriocin B2 was done with a 1% inoculum of an overnight culture of *C. divergens* LV13 containing pJKM14 grown in 2 liters of APT broth containing 5 μg of erythromycin per ml for 21 h maintained at pH 6.2 with a pH stat (Chem-Cadet; Cole Palmer). The culture was heated (70°C, 35 min), and cells were removed by centrifugation. Supernatant was loaded onto an Amberlite XAD-8 column (4 by 40 cm; BDH Chemicals, Poole, England) equilibrated with 0.05% trifluoroacetic acid. The column was washed successively with 3 liters each of 10, 35 and 40% ethanol. *C. divergens* LV13 containing pJKM14 produces carnobacteriocin B2 and divergicin A; hence, *C. divergens* LV13 was used as the sensitive indicator strain to eliminate inhibition by divergicin A. The active fraction was eluted with 3 liters of 50% ethanol. This fraction was concentrated by rotary evaporation to approximately 50 ml, and 10 ml was applied to a Sephadex G-50 column (2.5 by 120 cm; Pharmacia) with a running buffer of 0.05% trifluoroacetic acid. Contents of tubes with inhibitory activity were collected, pooled, and concentrated by rotary evaporation to 1 ml. Various amounts of partially purified carnobacteriocin B2 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane (Bio-Rad). A duplicate polyacrylamide gel was washed twice with 1 liter of water, and the gel was placed onto an APT plate and overlayered with soft APT agar inoculated with 1% *C. divergens* LV13. The band corresponding to inhibitory activity was excised from the membrane and used for N-terminal sequencing by Edman degradation as described by Worobo et al. (37).

TABLE 2. Bacteriocin production by strains of *Carnobacterium*

Producer strain	Inhibition zone diameter (cm) for indicator strain containing expression plasmid[a]					
	LV17C			LV13		
	pMG36e	pRW19e	pJKM14	pMG36e	pRW19e	pJKM14
C. piscicola						
LV17C(pMG36e)	0	0	0	0	0	0
LV17C(pRW19e)	30	0	30	0	0	0
LV17C(pJKM14)	6	6	0	20	20	0
C. divergens						
LV13(pMG36e)	23	0	23	0	0	0
LV13(pRW19e)	26	0	29	0	0	0
LV13(pJKM14)	24	6	24	19	19	0

[a] Zones of inhibition were determined by the deferred-antagonism assay.

RESULTS

Construction of plasmids pRW19e and pJKM14. The bacteriocinogenic plasmids pRW19e and pJKM14 were constructed for use in this study. These plasmids are derivatives of the lactococcal expression vector pMG36e (32), and transcription of the bacteriocin genes is under control of the P32 promoter. For construction of pRW19e, a 514-bp *Eco*RV-*Acc*I fragment of pCD3.4 containing both the structural and immunity genes for divergicin A (37) was cloned into the *Sma*I and *Acc*I sites of pMG36e. When *C. piscicola* LV17C was transformed with pRW19e the inhibitory spectrum matched that of *C. divergens* LV13 (Table 2). Zones of inhibition could be inactivated by pronase E. *C. piscicola* LV17C with pRW19e also acquired immunity to divergicin A (Table 2). For construction of pJKM14, a 528-bp fragment was amplified by PCR from pLQ24 using the primers JMc7 and ImmR. This fragment was cloned into the *Hin*dIII and *Xba*I sites of pUC118 to create the plasmid pJKM05 and sequenced in both directions to confirm the fidelity of the reaction. No errors were detected (data not shown) in the nucleotide sequence compared with nucleotide sequence of the structural and immunity genes for carnobacteriocin B2 (26). The 528-bp fragment was excised from pJKM05 with *Hin*dIII and *Kpn*I and cloned into these sites in pRW19e, replacing the divergicin A structural and immunity genes (Fig. 1). The *Sac*I-*Eco*RI fragment from pJKM14 containing the fusion between the sequence encoding the divergicin A signal peptide and the carnobacteriocin B2 structural gene was cloned into pUC118 and sequenced to confirm that the correct reading frame was maintained (data not shown).

Production of and immunity to divergicin A and carnobacteriocin B2. Bacteriocin production was detected by the deferred-inhibition assay and the spot-on-lawn test against sensitive indicator strains. Results of deferred-inhibition tests are shown in Fig. 2 and Table 2. *C. divergens* LV13 is more sensitive to carnobacteriocin B2 than *C. piscicola* LV17C, as shown by the large inhibitory zone in Fig. 2B. Zones of inhibition for wild-type strains and strains containing pMG36e were identical (data not shown). When *C. piscicola* LV17C was transformed with pRW19e, divergicin A was produced, as indicated by inhibition of strains sensitive to divergicin A. No activity against *C. divergens* LV13 was detected. Carnobacteriocin B2 from *C. divergens* LV13 and *C. piscicola* LV17C was assayed by the spot-on-lawn technique. In broth, the amounts of carnobacteriocin B2 produced are quite low. Only 100 activity units (AU) ml^{-1} were detectable after 21 h of growth from *C. piscicola* LV17C, and 200 AU ml^{-1} were detectable after 24 h of growth from *C. divergens* LV13. Strains containing pMG36e as controls did not produce inhibitory zones at any time. The wild-type carnobacteriocin B2 producer *C. piscicola* LV17B produces at least two bacteriocins (26), making comparisons between the inhibitory spectra of *C. piscicola* LV17B and *C. piscicola* LV17C containing pJKM14 difficult. To confirm the identity of the inhibitory substance produced by *C. divergens* LV13 containing pJKM14, the bacteriocins were purified and the N-terminal amino acid sequence of the probable carnobacteriocin B2 peak was determined and shown to be Val-Asn-Tyr-Gly-Asn-Gly-Val. This sequence matches the mature sequence of carnobacteriocin B2, indicating that the inhibitory substance was in fact carnobacteriocin B2 and that proper

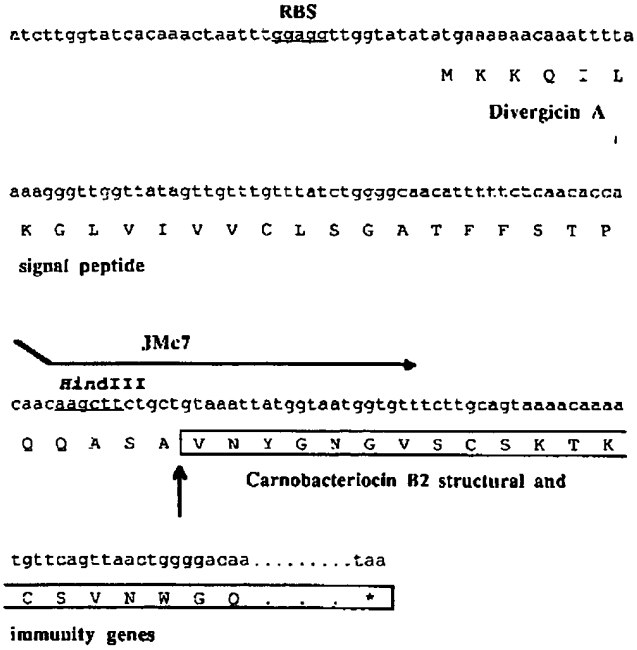

FIG. 1. Nucleotide and amino acid sequences of the divergicin A signal peptide fused to the product of the structural gene of carnobacteriocin B2 devoid of its natural leader peptide. The sequence for mature carnobacteriocin B2 is boxed. The locations of the forward primer (JMc7) used for PCR and the *Hin*dIII restriction endonuclease site are shown, and the signal peptide cleavage site is marked (↑). RBS, ribosome binding site.

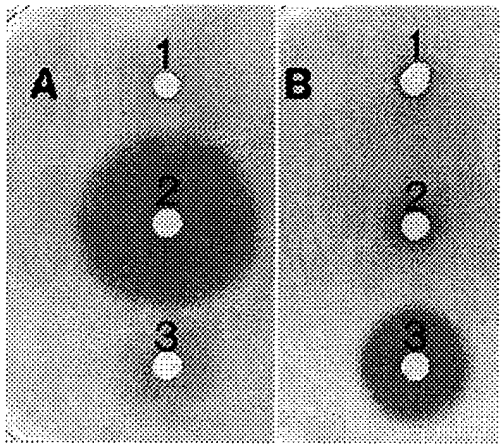

FIG. 2. Deferred-inhibition tests against *C. piscicola* LV17C (A) and *C. divergens* LV13 (B) with divergicin A and carnobacteriocin B2 by *C. piscicola* LV17C containing: 1, pMG36e; 2, pRW19e; 3, pJKM14.

processing of the bacteriocin occurred beyond the Ala-Ser-Ala processing site of the divergicin A signal peptide (Fig. 1). Production of carnobacteriocin B2 from pJKM14 was also accomplished in the meat isolates *C. piscicola* UAL26 and *C. divergens* UAL278 and in *Lactococcus lactis* IL1403 (data not shown).

DISCUSSION

The recent explosion of research on bacteriocins from LAB and other gram-positive bacteria resulted in the characterization of two novel bacteriocins, divergicin A and acidocin B, that appear to rely on the general protein secretion pathway in bacteria rather than the dedicated export systems of bacteriocins (21, 37). Acidocin B produced by *Lactobacillus acidophilus* has a relatively broad spectrum of activity and could have an important role in food safety; however, divergicin A produced by *C. divergens* does not inhibit organisms of practical importance. Antimicrobial activity of divergicin A has been demonstrated only against other species of *Carnobacterium* (37). *Carnobacteriocin* B2 is a relatively broad-spectrum bacteriocin with activity against many LAB, strains of *Listeria monocytogenes*, and some strains of *Enterococcus* spp. Our research goals include the development of LAB which produce multiple, broad-spectrum bacteriocins with different spectra of activity to encompass a broad range of antimicrobial activity. Using a general secretion pathway rather than a dedicated export system offers the advantage of a substantial decrease in the amount of genetic information required for independent bacteriocin expression. In the case of carnobacteriocin B2, approximately 9.5 kb of DNA is required (25). In this study we have reduced the amount of DNA required for expression of carnobacteriocin B2 to approximately 600 bp, not including a promoter.

Export systems for class II bacteriocins typically contain two proteins, an ABC exporter and an accessory protein of unknown function (8). The ability of carnobacteriocin B2 to be secreted via the SEC pathway was originally questioned, partly because of the unknown function of the accessory protein. In gram-negative-bacterium systems, the accessory protein is postulated to facilitate export of compounds through both the inner and outer membranes (7). Zhang et al. (38) showed that although colicin V can be secreted across the cytoplasmic membrane via a SEC-dependent pathway, secretion into the external medium is not achieved. The colicin V dedicated secretion system is therefore necessary for export across both membranes. This function of the accessory protein is not required for export of proteins from gram-positive cells. It is unlikely that this protein modifies the bacteriocin because carnobacteriocin B2 does not undergo posttranslational modification (26). For the export of bacteriocins in gram-positive bacteria, the accessory protein must either facilitate export of the bacteriocin across the cytoplasmic membrane in conjunction with the ABC exporter protein or aid in processing of the leader peptide, or both. This study confirms that the accessory protein is not required for production of active bacteriocin, so it must be involved in export, leader peptide recognition, and/or processing. Also, it was not known whether the natural leader peptide of carnobacteriocin B2 was required to maintain the bacteriocin in an inactive state and whether replacing it with the signal peptide of divergicin A would have a toxic effect on the producer organism. If this is the case, the divergicin A signal peptide may also function to maintain the bacteriocin in an inactive conformation.

The typical leader sequences of class II bacteriocins have a large amount of homology (15), and it may be possible to produce multiple bacteriocins with dedicated secretion machinery of a single bacteriocin. There is evidence that some class II bacteriocins can access dedicated secretion machinery from heterologous bacteriocins. Allison et al. (2) reported that both peptides of the two-component lactacin F utilize the bacteriocin secretion machinery of *C. piscicola* LV17. The N-terminal leader peptides of the carnobacteriocins and the lactacin F peptides have the highest similarity of class II bacteriocins, but it is not known if bacteriocins with less similarity in their leaders could access the export machinery of *C. piscicola* LV17 or the secretion machinery of other bacteriocins. Some reports suggest that one set of export machinery may not function adequately to export heterologous peptides. When the leader peptide of the lantibiotic nisin Z was replaced with that of the homologous lantibiotic subtilin, the subtilin leader peptide directed biosynthesis and export of pronisin Z but it was not cleaved, resulting in at least 200-fold-lower antimicrobial activity (19). Van Belkum and Stiles (31) showed that lactococcin A utilizes the secretion system for leucocin A to a limited extent, while mutants in the leucocin A secretion machinery could not be complemented with lactococcin A secretion apparatus.

It is not clear why bacteriocins have a dedicated export system when it appears from this study that with an appropriate signal peptide they can access the general SEC-dependent pathway of the cell. SEC-dependent secretion requires a protein that contains an N-terminal signal sequence with three distinct characteristics, including a positively charged amino terminus, a hydrophobic core, and a cleavage region (16). The posttranslationally modified class I bacteriocins may be unable to cross the cytoplasmic membrane via the SEC pathway because the lanthionine ring structures could maintain the peptide in a secretion-incompetent conformation. This should not apply to class II bacteriocins. There are some features of proteins which inhibit their secretion by the SEC pathway (20, 22, 35). A net positive charge at the N terminus of a protein may interfere with export because of interactions with the negatively charged phospholipids in the cell membrane or the effect of the positively charged membrane potential (5). However, there is evidence that this restraint is not as important in gram-positive bacteria as it is in gram-negative bacteria. Borchert and Nagarajan (4) showed that a wide variety of amino acids are tolerated at the +1 position in the SEC-dependent secretion of levansucrase from *Bacillus amyloliquefaciens*. Furthermore, leucocin A contains a lysine residue at the mature N terminus and export is still accomplished (unpublished data). Plantaracin C from *Lactobacillus plantarum* has lysine residues as four of the first five amino acids (12). It would be interesting to determine if multiple positive charges at the N termini of these bacteriocins will interfere with export via the divergicin A signal peptide secretion system. There may also be other sequences located within larger proteins which may inhibit export, but it has not been determined if this is simply due to features of protein folding (20, 28). Molecular folding of the small class II bacteriocins would not likely inhibit export, but it could affect the larger heat-labile class III bacteriocins, such as the chromosomally encoded, hydrophilic bacteriocin helveticin J (9, 17).

Bacteriocins may utilize the dedicated export pathway because it may be more efficient for the cell; however, comparison between these two systems would be very difficult. As shown here, under control of the P32 promoter in the plasmid pMG36e, export of carnobacteriocin B2 by the general protein secretion pathway appears to be less efficient in comparison with production from the wild-type organism. Production from *C. piscicola* LV17B reached a maximum of 400 AU/ml (26). *C. piscicola* LV17B produces at least two separate bacteriocins

from one set of secretion machinery which appears to be under the control of a complex regulation system (25). For comparison, the bacteriocin export cluster and the signal peptide dependent system should be expressed from the same expression vector to control for the copy number of these genes, and transcription should be under control of the same promoter. However, this would not take into account the amounts of the dedicated secretion proteins relative to the amounts of the SEC pathway proteins.

The signal peptide of divergicin A functions in a variety of hosts because carnobacteriocin B2 was also produced in the meat isolates *C. piscicola* UAL26 and *C. divergens* UAL278 and in *Lactococcus lactis* IL1403 (data not shown). Divergicin A is also produced by *E. coli* (37). Our goal is to use bacteriocin-producing LAB to inhibit pathogenic and spoilage organisms in meats and to use combinations of bacteriocins to enhance the overall effectiveness against target organisms. Using this system for bacteriocin export, we plan to express various bacteriocins that have different spectra of activity and possibly different modes of action from a single organism. Specific strains of LAB will be chosen for use as hosts for this vector on the basis of their characteristics relevant to certain food systems.

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A Signal Peptide Secretion-Dependent Bacteriocin from *Carnobacterium divergens*

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Divergicin A is a strongly hydrophobic, narrow-spectrum, nonlantibiotic bacteriocin produced by *Carnobacterium divergens* LV13. This strain of *C. divergens* contains a 3.4-kb plasmid that mediates production of, and immunity to, the bacteriocin. N-terminal amino acid sequencing of the purified divergicin A was used to locate the structural gene (*dvnA*). The structural gene encodes a prepeptide of 75 amino acids consisting of a 29-amino-acid N-terminal extension and a mature peptide of 46 amino acids. Directly downstream of *dvnA* there is a second open reading frame that encodes the immunity protein for divergicin A. Divergicin A has a calculated molecular mass of 4,223.89 Da. The molecular mass determined by mass spectrometry is 4,223.9 Da, indicating that there is no posttranslational modification of the peptide. The N-terminal extension of divergicin A has an Ala-Ser-Ala (positions −3 to −1) cleavage site and acts as a signal peptide that accesses the general export system of the cell (such as the *sec* pathway in *Escherichia coli*). This is the first bacteriocin of lactic acid bacteria to be reported that does not have dedicated maturation and secretion genes. Production of divergicin A was observed in heterologous hosts containing only the two genes associated with divergicin A production and immunity. Fusing alkaline phosphatase behind the signal peptide for divergicin resulted in the secretion of this enzyme in the periplasmic space and supernatant of *E. coli*.

Bacteriocins are antagonistic peptides or proteins that typically inhibit the growth of closely related bacteria. Klaenhammer (15) divided the bacteriocins of lactic acid bacteria (LAB) into four classes on the basis of their chemical, structural, and functional properties. The class I and II bacteriocins are the best characterized. They are small, heat-stable peptides that are inactivated by proteolytic enzymes of the intestinal tract. This makes them interesting as potential preservatives for foods. Class III and IV bacteriocins are not as well characterized and they are generally heat-labile, large proteins or protein conjugates, respectively. The class I bacteriocins are known as lantibiotics because they contain the unusual amino acids lanthionine and β-methyllanthionine. The lantibiotics are not exclusive to LAB, but they include nisin, which is an important food preservative produced by *Lactococcus lactis* subsp. *lactis* (8). Their production is characterized by extensive posttranslational modification of the gene product to produce the active peptide. Fusion of the N-terminal extension of subtilin in front of the structural component of nisin resulted in the production of nisin, suggesting the involvement of the N-terminal extension in the secretion of lantibiotics (17). Nisin has a polycistronic gene cluster that requires close to 10 kb of DNA to control its production, secretion, and posttranslational modification (16). Other LAB produce lantibiotics, such as carnocin UI49 produced by *Carnobacterium piscicola* (29), but these lantibiotics have not been extensively characterized.

Class II bacteriocins produced by LAB are the largest group that have been characterized. They are produced as prebacteriocins that comprise the precursor of the mature peptide with an 18- to 24-amino-acid N-terminal extension that is cleaved during secretion or maturation of the bacteriocin. They are minimally modified, apart from cleavage of the N-terminal extension at the Gly-Gly (positions −2 and −1) site. The role of the N-terminal amino acid extension of the class II bacteriocins has not been established, but it may be important in recognition of the prebacteriocin by the transport and maturation machinery of bacteriocin-dependent secretion systems. The N-terminal extensions of class II bacteriocins have marked homology in their hydrophobicity profiles (10, 13, 24). The class II bacteriocins, such as the lactococcins (28) and pediocin PA-1/AcH (21, 22) require dedicated secretion and maturation systems to produce the extracellular, mature bacteriocin. They require less genetic information than nisin for their production, but they generally have structural and immunity genes as an operon, with the secretion genes in close proximity in a relatively condensed gene package contained in approximately 3.5 to 4.5 kb of DNA (21, 31).

The focus of our research on bacteriocins of the meat-related LAB *Carnobacterium piscicola* (currently proposed as *Carnobacterium maltaromicus* [6]) and *Leuconostoc gelidum* (12, 24, 39) has been to characterize these bacteriocins with a view to enhancing their antibacterial spectrum through site-directed mutagenesis or production of two or more bacteriocins within gene cassettes. *C. piscicola* LV17 produces three bacteriocins (24, 39) and contains at least two independent secretion systems for the three bacteriocins, although the regulation of production of these bacteriocins is interrelated (27). In contrast, lactococcins A, B, and M share a common secretion system for all three bacteriocins (31). This indicates that the expression of multiple bacteriocins by one organism may require more than one secretion system and multiple immunities. There are no reports of immunity proteins capable of expressing immunity to more than one bacteriocin or of secretion proteins involved in the production of multiple, heterologous bacteriocins. As a result of these bacteriocin-dependent immunity and secretion genes, gene cassettes would be difficult to produce with class II bacteriocins because of the large amount of DNA required for immunity to, and production of, each bacteriocin. In this paper we report the purification and sequence of a novel bacteriocin, divergicin A, that functions as

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TABLE 1. Bacteria and plasmids used to study bacteriocin production by *C. divergens* LV13

Bacterial strain or plasmid	Description[a]	Source or reference
C. divergens		
LV13	*dvn*$^+$ *dvi*$^+$ (containing pCD3.4)	NCFB[b]
AJ	Dvn^s (indicator strain)	Laboratory isolate
C. piscicola		
LV17C	Dvn^s (indicator strain), plasmidless	2
LV17A	*cbnA* (containing pCP49), Bac$^+$ Dvn^s	2
LV17B	*cbnB2* and *cbnBM1* (containing pCP40)	2
UAL 26	Bac$^+$ Dvn^s	2
L. lactis subsp. *lactis*		
MG1363	Dvn^r, plasmidless	11
IL1403	Dvn^r, plasmidless	5
E. coli		
DH5α	F$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1* Δ(*argF-lacZYA*)UI69 ϕ80*dlac* ZΔM15	Bethesda Research Laboratories Life Technologies Inc.
MH1	MC1061 derivative; *araD139 lacX74 galU galK hsr hsm*$^+$ *strA*	4
Plasmids		
pCD3.4	*dvn*$^+$ *dvi*$^+$, 3.4 kb	This study
pCD4.4	pCD3.4 containing 1.0-kb *Eco*RI Cm^r gene of pGS30; Cm^r *dvn*$^+$ *dvi*$^+$, 4.4 kb	This study
pUC118	*lacZ'* Amp^r, 3.2 kb	34
pGS30	pUC7 containing 1.0-kb *Pst*I Cm^r gene of pC194; Cm^r, 3.7 kb	G. Venema[c]
pKM1	pUC7 containing 1.3-kb *Pst*I Km^r gene of pUB110; Km^r, 3.7 kb	G. Venema[c]
pGKV259	Em^r Cm^r 5.0 kb	33
pRW5.6	pGKV259 containing 514-bp *Eco*RV-*Acc*I fragment; Em^r *dvn*$^+$ *dvi*$^+$, 5.6 kb	This study
pRW6.0	pGKV259 containing divergicin signal peptide fused to alkaline phosphatase	This study

[a] *dvn*$^+$, divergicin production gene; *dvi*$^+$, divergicin immunity gene; Dvn^r, divergicin resistant; Dvn^s, divergicin sensitive; Bac$^+$, bacteriocinogenic; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Km^r, kanamycin resistance.

[b] NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

[c] Strain from the laboratory of G. Venema, Department of Genetics, University of Groningen, Haren, The Netherlands.

a signal peptide secretion-dependent bacteriocin, which does not require bacteriocin dedicated secretion machinery.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Carnobacteria were grown in APT broth (Difco Laboratories Inc., Detroit, Mich.) at 25°C, and *Escherichia coli* was grown in Luria-Bertani (LB) broth (26) on a rotary shaker at 250 rpm at 37°C. Solid media were prepared by adding 1.5% agar to the broth media; overlay media were prepared with 0.75% agar. Stock cultures of carnobacteria were maintained at 4°C in Cooked Meat Medium (Difco) and at −70°C in APT broth containing 40% (vol/vol) glycerol. *E. coli* strains were stored in LB broth with 40% (vol/vol) glycerol at −70°C. When appropriate, media were supplemented with antibiotics in the following concentrations: ampicillin and erythromycin at 200 μg/ml for *E. coli* and erythromycin at 10 and 5 μg/ml for broth and solid media, respectively, for carnobacteria.

Production and purification of divergicin. Partial purification of divergicin was done with a 1% inoculum of an overnight culture of *Carnobacterium divergens* LV13 in 2 liters of APT broth, which was maintained at pH 7.5 with 2 N NaOH by using a pH stat (Chem-Cadet; Cole Palmer) while being stirred gently at 25°C. The culture was adjusted to pH 5.6 with 5 N HCl and heated to 70°C for 35 min before centrifugation (9,000 × *g*, 5 min, 4°C). The bacteriocin in the supernatant was precipitated with ammonium sulfate (700 g/liter) by stirring for 24 h at 4°C and was harvested by centrifugation (9,000 × *g*, 20 min, 4°C). The precipitate was dissolved in 60 ml of 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma Chemical Co., St. Louis, Mo.), pH 5.5 (fraction A). Ammonium sulfate (200 g/liter) was added to fraction A, and precipitated proteins were removed by centrifugation (9,000 × *g*, 20 min, 4°C). The clarified fraction A was loaded onto a 45-ml octyl-Sepharose CL4B chromatography column (Pharmacia, Uppsala, Sweden) equilibrated with 20% ammonium sulfate in 20 mM MES, pH 5.5. The column was washed with 1.5 volumes each of 20, 15, 10, 5, and 0% ammonium sulfate in 20 mM MES, pH 5.5, and then with 1.5 volumes each of water, 10% ethanol, and 70% ethanol. The active fraction was concentrated by rotary evaporation, resuspended in 6 M urea, and loaded onto a Sephadex G-75 column (2.5 by 120 cm; Pharmacia) equilibrated with 6 M urea. Elution was monitored by A_{228}. All fractions were assayed for bacteriocin activity by the spot-on-lawn test (2). The urea was dialyzed in 3,500-molecular-weight-cutoff dialysis tubing (Spectra Por; Spectrum Medical Industries Inc., Los Angeles, Calif.) and lyophilized.

Complete purification was achieved by using the method previously described by Hastings et al. (12), except that the culture was grown in 5 liters of APT broth supplemented with 1% glucose. After incubation for 20 h, the cells were removed by centrifugation and the culture supernatant was applied directly to an Amberlite XAD-8 column (4 by 40 cm; BDH Chemicals Ltd., Poole, England) that was washed with 3 liters of 0.05% trifluoroacetic acid (TFA) and 2 liters each of 20 and 35% ethanol in 0.05% TFA. The active fraction eluted with 50% ethanol in 0.05% TFA (1.5 liters) was concentrated by rotary evaporation, and 10% (5 ml) was loaded onto a Sephadex G-50 column (2.5 by 120 cm; Pharmacia) that had been equilibrated with 0.05% TFA. The active fractions were concentrated to 1 ml by rotary evaporation, applied in 100-μl portions to a C_4 column (Waters Delta-Pak; 10 by 200 mm; 15 μm particle size; 300 Å [30 nm] pore size flow rate, 1.5 ml/min; mobile phase, 0.05% TFA in water [A] and 95% ethanol in 0.05% TFA [B]), and eluted by a gradient method (first 50 to 63% solvent B in 7 min and then 63 to 64% in 6 min). Fractions were monitored for A_{218} and for activity against the indicator strain. The purities of the fractions were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Bacteriocin production and immunity were tested by the deferred inhibition assay (2).

SDS-PAGE. Purified divergicin preparations were examined for the presence of contaminating proteins by using 15% polyacrylamide gels and 3 M Tris-HCl, pH 8.8. Electrophoresis was done at constant current of 20 mA as described by Laemmli (18). Gels were fixed in 50% (vol/vol) methanol–10% (vol/vol) acetic acid for 30 min and stained with Coomassie brilliant blue (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). To test for activity, the polyacrylamide gel was washed with water (twice with 1 liter each time) for 2.5 h. The gel was placed onto an APT plate and overlayered with soft APT agar inoculated with 1% of a sensitive indicator strain.

N-terminal amino acid sequence and amino acid analysis. Partially purified divergicin obtained by using octyl-Sepharose CL4B and Sephadex G-75 was subjected to SDS-PAGE, and the proteins were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) at 50 V for 3 h in blotting buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3 [pH 9.9] in 20% ethanol [9]). The blotted membrane was stained with 0.25% Coomassie brilliant blue in 40% methanol and destained with 50% methanol. The protein band corresponding to activity on the overlayered gel was excised and used for N-terminal amino acid sequencing by Edman degradation analysis on an automated gas phase sequencer (model 470A; Applied Biosystems, Foster City, Calif.) with on-line phenylthiohydantoin derivative identification by reversed-phase high-pressure liquid chromatography (HPLC) (Applied Biosystems model 120A). Amino acid analysis was done on a similarly excised band containing the peptide. The amino acids were derivatized with phenylisothiocyanate on an Applied Biosystems (model 420A) derivatizer and separated by HPLC (Applied Biosystems model 130A) with a C_{18} column. Data were recorded and analyzed on an Applied Biosystems (model 920A) data analyzer system.

Mass spectrometry. The mass spectra of divergicin were determined by Paul Semchuk of the Protein Engineering Centres of Excellence, University of Victoria, Victoria, British Columbia, Canada. Electrospray mass spectra were acquired on a Fisons VG Quatro instrument. Divergicin, which had been purified by HPLC, was introduced by direct infusion (1 μg/ml) in 50% aqueous acetonitrile containing 0.5% TFA through a sample loop. Fisons software was employed to determine the molecular mass from the envelopes of multiply charged peaks in the *m/z* spectra.

DNA isolation, manipulation, and sequence analysis. Large-scale plasmid preparation from *C. divergens* LV13 was done as previously described for *C. piscicola* LV17A (39). Other DNA manipulations were based on those described by Sambrook et al. (26). *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and restriction endonucleases and T4 DNA ligase (Promega, Madison, Wis.; Bethesda Research Laboratories, Burlington, Ontario, Canada; Boehringer Mannheim, Dorval, Quebec, Canada; and New England Biolabs, Mississauga, Ontario, Canada) were used according to the suppliers' recommended procedures. Stepwise deletion derivatives for sequencing were prepared by using the Erase-a-Base system (Promega), and DNA fragment recovery was done with Geneclean II (Bio 101 Inc., La Jolla, Calif.). Carnobacteria were transformed by resuspending a 3-h culture from a 2% inoculum in 1/10 of the volume of cold Milli-Q water and collecting the cells by centrifugation at 6,000 × *g*. The cells were washed twice with electroporation buffer (0.5 M sucrose, 2.5 mM $CaCl_2$) and resuspended in 1/40 of the original volume of electroporation buffer. The cells were electroporated with a Gene Pulser (Bio-Rad) by the method recommended by the supplier. Immediately following electroporation, 1 ml of APT containing 0.5 M sucrose was added, and the cells were incubated at room temperature for 3 h and plated onto appropriate selective media. Oligonucleotides prepared as sequencing and PCR primers were synthesized on an Applied Biosystems (model 391) PCR Mate synthesizer. Double-stranded DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing on an Applied Biosystems (model 373A) sequencer. For amplification of the DNA encoding the mature part of alkaline phosphatase, primers KLR 179 (5′ GCGCAAGCTTCTGCTCGGACACCAGAAATGCCTGTT 3′) and KLR 180 (5′ GGCCAAGCTTGCCATTAAGTCTGGTTGCTA 3′) were used with the *E. coli* C_4F_1 (30) alkaline phosphatase gene as a template.

Assay for alkaline phosphatase. Cells from 1.5 ml of an overnight culture grown in LB broth were centrifuged (9,000 × *g*, 5 min, 25°C) and washed in an equal volume of STE (50 mM NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]). The culture medium and periplasmic fractions were assayed for alkaline phosphatase. Periplasmic fractions were prepared by resuspending the washed cells in 0.5 ml of 20% sucrose with 50 μl of 0.5 M EDTA and 25 μl of lysozyme (10 mg/ml) and incubating at room temperature for 15 min. The samples were centrifuged (9,000 × *g*, 5 min, 25°C), and the supernatant was assayed for alkaline phosphatase activity (30) by A_{405}.

Nucleotide sequence accession number. The DNA sequence was submitted to GenBank (Los Alamos, N.Mex.) and was given the accession number L37791.

RESULTS

Production and purification of divergicin. *C. divergens* LV13 produces a bacteriocin, divergicin A, that is active against the indicator strains *C. piscicola* LV17C and *C. divergens* AJ. The amount of divergicin produced by *C. divergens* LV13 in APT broth at pH 7.5 and 25°C for 18 h is 2 mg/liter. Different media and pH conditions were tested for increased bacteriocin production. Divergicin was not produced in the semidefined medium CAA used by Hastings et al. (12). Of the media tested, only APT supported bacteriocin production. The best production was achieved at pH 6.5 with 1% glucose added to the APT broth. For purification of divergicin by ammonium sulfate precipitation followed by octyl-Sepharose chromatography, all of the activity was eluted in the 70% ethanol wash. With size exclusion chromatography (Sephadex G-75), most of the activity was detected in the second peak. This fraction was concentrated, subjected to SDS-PAGE, and blotted onto a polyvinylidene difluoride membrane. The band corresponding to activity on the SDS-PAGE gel that was overlayered with the sensitive *C. piscicola* LV17C indicator strain was excised from the membrane and used for Edman degradation and amino acid analysis. Divergicin is rapidly inactivated in acetonitrile, which was used in our initial HPLC purification protocol, but it was stable in ethanol. The use of ethanol as the carrier solvent enabled further purification of divergicin A and determination of its molecular mass. HPLC-purified bacteriocin for mass spectrometry was obtained by using the protocol of Hastings et al. (12) with gradients of ethanol and water containing TFA to elute the pure divergicin.

N-terminal sequence and amino acid analysis of divergicin. The excised bacteriocin band from the electroblotted polyvinylidene difluoride membrane enabled the following 21-amino-acid N-terminal sequence to be determined: Ala-Ala-Pro-Lys-Ile-Thr-Gln-Lys-Gln-Lys-Asn-X-Val-Asn-Gly-Gln-Leu-Gly-Gly-Met-Leu-Ala. This sequence matches the amino acid content derived from the nucleotide sequence and amino acid analysis except for the apparent absence of methionine in the amino acid analysis. Methionine was not detected in the amino acid analysis, probably because of the small amount of bacteriocin contained on the blotted membrane. The unidentified amino acid at position 12 was subsequently shown to be cysteine by interpretation of the nucleotide sequence (Fig. 1).

Mass spectrometry of divergicin. Mass spectral analysis by positive-ion fast atom bombardment with an electrospray interface gave multiply charged molecular ions (Fig. 2), which allowed the calculation of an average molecular mass of 4,223.9 ± 0.1 Da.

Identification and expression of the divergicin structural and immunity genes. The native plasmid pCD3.4 was completely sequenced (unpublished data), and three open reading frames (ORFs) were identified. The N-terminal amino acid sequence of divergicin was observed in the nucleotide translation product in the ORF containing 225 nucleotides. This ORF is contained in the 514-bp *Eco*RV-*Acc*I fragment of pCD3.4 (Fig. 1), starting with the alanine residue at position 30 of the structural gene for divergicin A (*dvnA*). This gene encodes a 75-amino-acid prepeptide consisting of a 29-amino-acid N-terminal extension and a 46-amino-acid bacteriocin. Immediately following the divergicin structural gene is a second ORF that encodes 56 amino acids that could be the protein for divergicin immunity. Two probable ribosome binding sites (GGAGG) for the divergicin structural gene and the possible divergicin immunity gene are located 9 and 13 bp, respectively, upstream of the initiator codons. Downstream of the second ORF (nucleotides 530 to 564) there is a 14-base inverted repeat with a 7-base loop that is a potential *rho*-independent terminator. A unique *Ssp*I site contained within the divergicin immunity gene (*dviA*) was used to insert a kanamycin resistance marker from pKM1 and to inactivate the immunity gene. Nucleotide sequencing of the immunity gene was done to confirm insertion into the *dviA* gene. The loss of immunity was accompanied by the strain still producing divergicin A, but growth of these organisms was poor.

The native plasmid pCD3.4 with a chloramphenicol resistance marker from pGS30 inserted into the *Eco*RI site (not shown) to produce pCD4.4 was used to transfer the plasmid to heterologous carnobacterial hosts, including *C. piscicola* LV17A, LV17B, LV17C, and UAL 26 (Table 1). Transformation of pCD4.4 into heterologous *C. piscicola* and *C. divergens* hosts resulted in chloramphenicol resistance as well as in production of, and immunity to, divergicin. To utilize the *Pst*I site immediately following the P59 promoter of pGKV259, the 514-bp *Eco*RV-*Acc*I fragment and the *Pst*I site of pGKV259 were blunt ended and the fragment was cloned into the *Pst*I site, resulting in the construct pRW5.6. The presence of the correct fragment and orientation for the expression of divergicin A was confirmed by restriction enzyme analysis. The presence of the structural and possible immunity genes for divergicin was also confirmed by nucleotide sequencing. Electroporation of the pRW5.6 construct into other *Carnobacterium* hosts resulted in full divergicin production and immunity as determined by deferred inhibition assays (Fig. 3). The "half moon" halo effect in the zones of inhibition shown in Fig. 3B

FIG. 1. Nucleotide sequence of a 581-bp fragment from pCD3.4, a native plasmid of *C. divergens* LV13. The deduced amino acid sequences for the precursor of divergicin A and its putative immunity protein are shown below the nucleotide sequence. A potential *rho*-independent termination site is indicated by reversed arrows on the nucleotide sequence, and the putative ribosome binding site (RBS) is underlined.

was created by spotting pronase E in close proximity to the producer organism, confirming that the inhibition was due to bacteriocin production. The pRW5.6 construct was also electroporated into two *Lactococcus* strains, MG1363 and IL1403. Expression was achieved in both of these strains, with more divergicin being produced by strain MG1363 than by strain IL1403. Divergicin immunity in the *Lactococcus* strains could not be tested because the host strains are normally resistant to divergicin.

Alkaline phosphatase fusion. PCR-generated DNA encoding the mature part of alkaline phosphatase with *Hin*dIII linking ends was inserted into the *Hin*dIII site of the divergicin signal peptide. The *dvnA* Ala-Ser-Ala cleavage site was regenerated by incorporation of these three residues into the PCR primer. The presence of phosphatase activity was screened by direct plating of the ligation mixture onto LB agar containing 5-bromo-4-chloro-3-indolyl phosphate and appropriate antibiotics. Blue colonies were selected, and the transformants were cultured in liquid medium. The periplasmic and culture medium fractions were assayed for phosphatase activity by a spectrophotometric method (30) that uses the hydrolysis of *p*-nitrophenylphosphate and the production of *p*-nitrophenol for quantitative assay. Activities (micromoles of *p*-nitrophenol per minute per milliliter of original culture) were as follows: *E. coli* MH1 supernatant, 4.37; MH1 periplasmic fraction, 0.18; MH1/pRW6.0 supernatant, 167.00; and MH1/pRW6.0 periplasmic fraction, 89.35. These data show that the alkaline phosphatase activity for the phosphatase fusion is located in the culture medium and the periplasm. The amount of phosphatase activity in the fusion construct compared with that in the control shows active production and secretion of phosphatase.

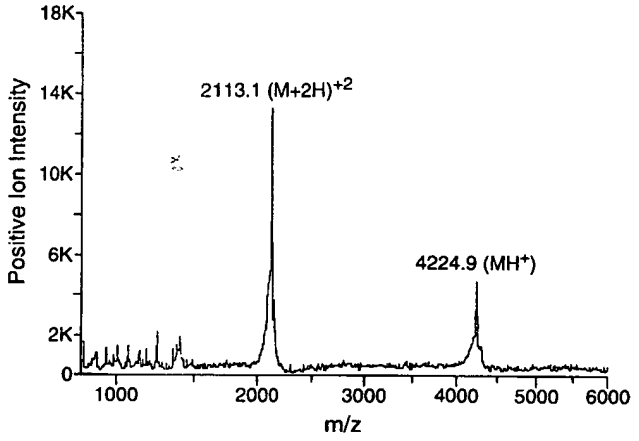

FIG. 2. Electrospray fast atom bombardment mass spectrum of divergicin A, showing multiply charged molecular ions from which the atomic mass was calculated.

DISCUSSION

We are interested in the development of a food grade vector suitable for use in our studies of bacteriocin production by LAB that were isolated from meats. The plasmid pCD3.4 in *C. divergens* LV13 was of interest because of its high copy number and the possibility that it was responsible for the production of divergicin A. Initially it was thought that the plasmid was too small to contain the genetic information for bacteriocin production or that bacteriocin production was controlled by genes on the plasmid and on the chromosome, as in the case of carnobacteriocin BM1 (25). The purification of divergicin A and the determination of its N-terminal sequence enabled the position of the structural gene for divergicin to be located on pCD3.4. From the precise agreement of the calculated and determined molecular masses of divergicin A of 4,223.89 and

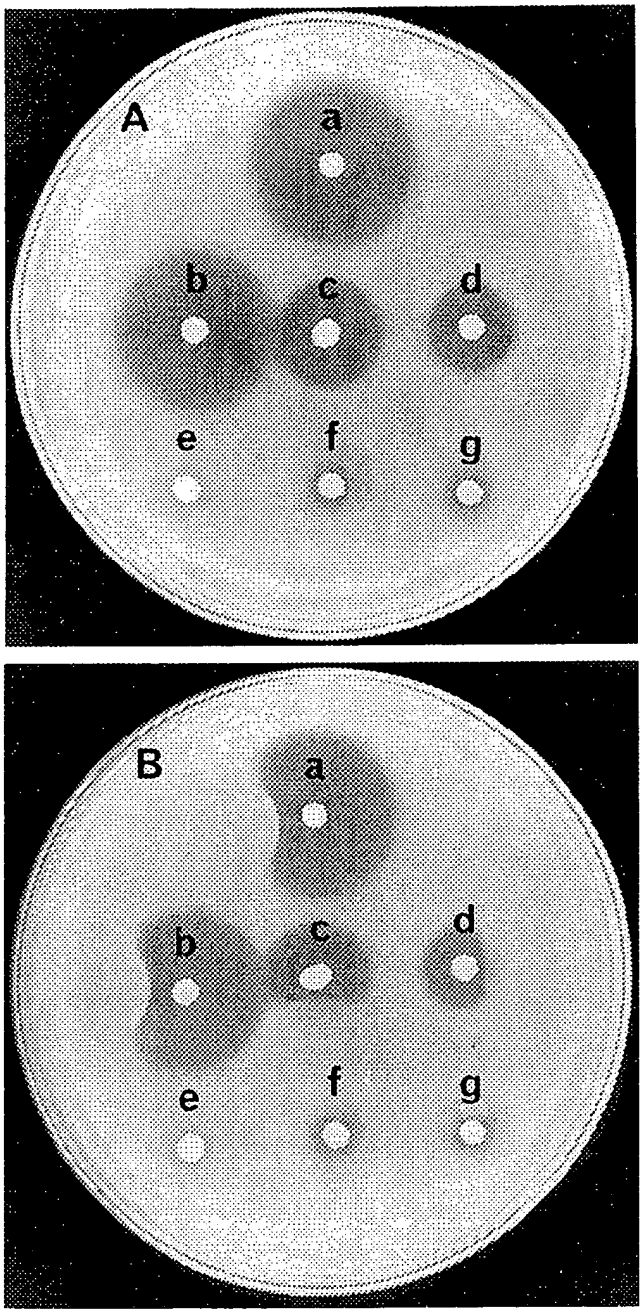

FIG. 3. Deferred inhibition of *C. divergens* AJ by homologous and heterologous hosts transformed with the divergicin A gene. (A) a, *C. divergens* LV13; b, *C. piscicola* LV17C containing pRW5.6; c, *L. lactis* MG1363 containing pRW5.6; d, *L. lactis* IL1403 containing pRW5.6; e, plasmidless *C. piscicola* LV17C; f, plasmidless *L. lactis* MG1363; g, plasmidless *L. lactis* IL1403. (B) Inactivation of divergicin A by pronase E. The producer strains are as described for panel A.

4,223.9 Da, respectively, it was evident that no posttranslational modifications occurred, such as a disulfide bridge between the cysteine residues at positions 12 and 44. The formation of disulfide bridges in mature bacteriocins has been observed for carnobacteriocins A and B2 (24, 39), leucocin A (12), and pediocin PA-1 (21).

Divergicin A has a novel amino acid sequence, N-terminal extension, and processing site. Its small size, hydrophobic nature and thermostability are indicative of a class II bacteriocin

N-terminal extension of class I & II bacteriocins

Class I	
Nisin A	MSTKDFNLDLVSVSKKDSGASPR-
Class II	
Pediocin PA-1	MKKIEKLTEKEMANIIGG-
Leucocin A	MMNMKPTESYEQLDNSALEQVVGG-
Carnobacteriocin A	MNNVKELSIKEMQQVTGG-

Signal peptide of divergicin A

MKKQILKGLVIVVCLSGATFFSTPQASA-

FIG. 4. Amino acid sequence of the signal peptide of divergicin A compared with the sequences of the N-terminal amino acid extensions of well-characterized class I and class II bacteriocins of LAB. The sequences for nisin A, pediocin PA-1, leucocin A, and carnobacteriocin A are from references 16, 21, 12, and 39, respectively.

as defined by Klaenhammer (15). This class of bacteriocins includes pediocin PA-1; sakacins A and P; curvacin A; lactacin F; lactococcins A, B, G, and M; carnobacteriocins A, BM1, and B2; mesentericin Y105; and leucocin A-UAL 187. All of the class II bacteriocins for which the nucleotide sequence has been characterized are produced as precursors that contain an N-terminal extension with a Gly-Gly processing site at positions −1 and −2 of the cleavage site. These N-terminal extensions have marked homology in their amino acid sequences (10, 13, 15, 24). However, the N-terminal extension of divergicin has no homology with that of the class II bacteriocins, and, compared with other LAB bacteriocins, it has a unique processing site of Ala-Ser-Ala. No homology exists with the recently identified peptide antibiotic AS-48, produced by *Enterococcus faecalis*, which is postulated to contain a signal peptide in the N-terminal region of the immature antibiotic (20). The N-terminal extension of the divergicin structural gene resembles a signal peptide (Fig. 4), comparable to signal peptides of α- and β-amylases, alkaline phosphatase, outer cell wall proteins, β-lactamase, fimbriae, and proteases that access the *sec*-dependent pathway in bacteria (38). Signal peptides have three distinct domains referred to as the N, C, and H regions (23, 38). The N terminus region has at least one lysine residue in the first 6 to 8 amino acids, the H region contains 8 to 15 amino acids and has strong hydrophobicity, and the C region contains the cleavage site that consists of neutral amino acids with small side chains at positions −3 and −1, respectively (35, 36). The 29-amino-acid N-terminal region of divergicin complies with all of the Von Heijne rules for a signal peptide. The signal peptide for divergicin also contains a proline located two residues upstream of the Ala at position −3. This is thought to bend the signal peptide so that it can expose the cleavage site to the peptidase (37).

Class I and II LAB bacteriocins that have been characterized require a dedicated secretion apparatus that in most cases has been shown to be genetically linked to, or associated with, the bacteriocin structural and immunity genes. The possibility that all of the genetic information required for divergicin expression was contained on a small 3.4-kb plasmid seemed likely because transformation of the pCD3.4 plasmid into heterologous, bacteriocinogenic and nonbacteriocinogenic hosts resulted in divergicin production and immunity. The remaining 2.9-kb portion of pCD3.4 is too small to encode the different proteins necessary for secretion of the bacteriocin equivalent to those for pediocin PA-1, lactococcins A, B, and M, or nisin A. All of these bacteriocins rely on two or more large proteins for their activity, including an ATP binding cassette translocator protein (15).

Comparison of the N-terminal sequence with those of established signal peptides and the absence of additional secretory and maturation genes indicated that divergicin may be utilizing existing signal sequence-dependent general export pathways in the host. This was confirmed by cloning the dedicated genes for divergicin production and immunity present on the 514-bp *Eco*RV-*Acc*I fragment of pCD3.4 behind the P59 promoter of pGKV259. Production of divergicin A was also observed in heterologous hosts such as *C. piscicola* LV17C and *L. lactis* IL1403 and MG1363. *L. lactis* IL1403 contains a set of genes on the chromosome that are involved in production and secretion of lactococcins (28). However, production of divergicin in IL1403 was not greater than that observed in MG1363, indicating that a dedicated bacteriocin secretion machinery is probably not involved in divergicin production. Conclusive evidence that the N-terminal extension of divergicin acts as a signal peptide was achieved with the production of alkaline phosphatase in *E. coli* when the native signal peptide was replaced by that of divergicin. Divergicin requires as little as 0.5 kb for its production, whereas most bacteriocins require 3.5 to 10 kb for independent bacteriocin production. Although the spectrum of activity for divergicin A is narrow, the signal peptide creates interesting possibilities for secreting other bacteriocins via the *sec*-dependent pathway. With fusions between the signal peptide gene of divergicin and structural genes of other bacteriocins, it may be possible for bacteriocins of interest to be secreted without the specific secretion and maturation proteins.

Most LAB bacteriocins have a regular pattern of hydrophobic and hydrophilic domains. Divergicin A is a very hydrophobic molecule, with a calculated pI of 9.2 and only one small hydrophilic region. Several class II bacteriocins have been shown to contain a positively charged residue at the N terminus of the mature molecule. This would most likely inhibit secretion through the general export pathway (14). The class II bacteriocins have a common mode of action (1, 3, 19, 32). They are membrane-active compounds that cause cell permeabilization with loss of proton motive force and efflux of intracellular components. The immunity protein of divergicin has the unusual feature of being a small, very hydrophobic molecule and has an unusual topology because the second of the two transmembrane segments is flanked by positive charges. Divergicin A may have a different mode of action or immunity. Divergicin A contains six Gly-Gly paired residues in the mature peptide. The Gly-Gly motif is a feature of microcin B17, which is an inhibitor of DNA replication (7). The unique natures of the Ala-Ser-Ala processing site and the signal peptide indicate that divergicin A does not fit into established classes of bacteriocins. Further study will indicate whether divergicin A represents a fifth class of bacteriocins (15). The detection of a similar system in *E. faecalis* (20) represents another bacteriocin that may access the *sec* pathway of bacteria.

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